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Big impacts by small RNAs in plant development

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The identification and study of small RNAs, including microRNAs and *trans*-acting small interfering RNAs, have added a layer of complexity to the many pathways that regulate plant development. These molecules, which function as negative regulators of gene expression, are now known to have greatly expanded roles in a variety of developmental processes affecting all major plant structures, including meristems, leaves, roots, and inflorescences. Mutants with specific developmental phenotypes have also advanced our knowledge of the biogenesis and mode of action of these diverse small RNAs. In addition, previous models on the cell autonomy of microRNAs may have to be revised as more data accumulate supporting their long distance transport. As many of these small RNAs appear to be conserved across different species, knowledge gained from one species is expected to have general application. However, a few surprising differences in small RNA function seem to exist between monocots and dicots regarding meristem initiation and sex determination. Integrating these unique functions into the overall scheme for plant growth will give a more complete picture of how they have evolved as unique developmental systems.

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Introduction

Since the last review in this journal of the role of microRNAs in development in plants [1], several advances in small RNA biology have been made, many novel and unexpected. This review focuses on how small RNAs have impacted our views of the development of several plant-specific structures in both monocots and dicots. While small RNAs have long been implicated in the determination of meristem boundaries in dicots, new data in monocots show that they are also essential for meristem initiation and maintenance. Small RNAs are known to control the morphogenesis and abaxial/adaxial patterning of leaves, but also have been shown to affect the differentiation of specific cells types, such as the stomata, and the development of serrations. Two microRNAs known to control developmental timing have been shown to participate in a wider range of functions, including light response, control of plastochron length, and sex determination. The sequential expression patterns of this pair of plant-specific microRNAs are reminiscent of those of temporally regulated microRNAs in animals, indicating potential convergence of timing mechanisms between the two kingdoms. Finally, more evidence supporting the long distance transport and function of microRNAs has accumulated, a fact that will have to be incorporated into future models for microRNA action.

Meristems

In plants, organogenesis occurs from meristems, groups of self-organizing cells whose derivatives divide and elongate, making up the cells of the plant body. The root and shoot apical meristems form early in the embryo and produce opposite poles of growth and differentiation. Additional meristems such as lateral root meristems or axillary meristems form during the life of the plant, helping to create the overall architecture.

Shoot meristems have two major functions; they maintain a population of totipotent cells and produce lateral organs whose fates are determined by genetic and developmental factors. During early vegetative phases, the lateral organs are leaves that may have axillary meristems in the region between the leaf and the stem. In addition, leaves initiated during the juvenile vegetative phase have different morphology and cell identities compared to those made in the adult phase. Later, during reproductive development, the leaves are smaller or suppressed altogether, and the axillary meristems grow out into flowers or branches. Regardless of their size, leaves arise with an inherent polarity. The branches, on the other hand, are usually radially symmetric. Meristems are classified as determinate or indeterminate based on whether they are consumed in the production of primordia. Floral meristems are determinate since they terminate growth after the initiation of floral organs, while branch meristems are usually indeterminate.

Several interesting mutants have been described in rice that either lack or fail to properly maintain the shoot meristem. The *shootless* (*shl*) mutants initiate the coleoptile and scutellum, which together form the cotyledon in the grasses [2], but fail to make the shoot meristem [3]. Weak alleles show that *SHL* genes are also required for

maintenance of the meristem [4]. The *shoot meristem organization* (*sho*) mutants produce radialized leaves with irregular phyllotaxy [5], reminiscent of the maize mutant *leafbladeless1* (*lbl1*), which also produces radialized leaves with altered *KNOX* gene expression [6].

The *sho* and *shl* mutants of rice and the *lbl1* mutant of maize are defective in small RNA processing. *SHL2* encodes an RNA-dependent RNA polymerase 6, *SHO2* encodes an AGO7, *SHO1* encodes DCL4 [7••], and *LBL1* encodes a SUPPRESSOR OF GENE SILENCING3 (SGS3) protein [8••]. All of these proteins are components of the si-RNA pathway [9] and, interestingly, the mutant phenotypes are more severe in maize and rice than in *Arabidopsis*, where they mainly affect phase change. In the *sho*, *shl*, and *lbl1* mutants, the expression of class III *HD-ZIP* genes (*HD-ZIPIII*) is decreased, and the expression of the *MIR166* microRNA is increased [7••,8••]. A critical role for these two components was shown by overexpressing a microRNA-resistant version of the *HD-ZIPIII* gene *OSHB1* in *sho1* mutants [7••]. The phyllotaxy was restored to normal and the plants no longer produced radial leaves. Two other key components are likely to be the *AUXIN RESPONSE FACTOR* (*ARF*) genes and the *trans*-acting small interfering RNAs that target them, *tasiR-ARFs*. Levels of *tasiR-ARFs* are decreased in both *lbl1* mutants and *DCL4* knockdowns, with a corresponding increase in specific *ARF* target genes [8••,10•]. The unanswered question is how the regulation of *ARFs* by *tasi-RNAs* and *HD-ZIPIII* genes by *MIR166* are connected. Perhaps *MIR166* is positively regulated by *ARF* transcription factors, or, there exists an unknown *tasi-RNA* that negatively regulates the precursor of *MIR166*.

The *cup-shaped cotyledon* (*cuc*) mutant of *Arabidopsis* was identified by an embryo defect in which cotyledons are fused and leaves fail to initiate [11]. The redundant *CUC1* and *CUC2* genes are expressed in domains of the embryo that demarcate the position of the cotyledons, suggesting that they function to repress growth in that region [12,13]. Because the *cuc1 cuc2* phenotype is seedling lethal and there is functional redundancy with *CUC3* [14,15], the postembryonic role of *CUC* genes has not been well studied. A series of papers explores how the *MIR164* gene family modulates the expression of *CUC* genes and helps to tease out specific functions for them at different times.

Mutations in *MIR164c*, but not *MIR164b*, produce extra petals on the first formed flowers [16]. This mild phenotype is enhanced in the *MIR164a MIR164b MIR164c* triple mutant [17•]. In fact, most flowers of the triple mutant also have extra sepals, fewer stamens, and highly variable organ number and organ size. Floral organ number is also changed in plants that express a microRNA-resistant version of *CUC2* [18]. *CUC2* also participates in leaf development, phyllotaxy, and axillary meristem development. Mutations in *MIR164a* as well as overexpression of microRNA-resistant versions of *CUC2* cause exaggerated leaf serrations. The serrations initiate as in wild-type but become enhanced due to growth repression by *CUC2* in the sinus region. Conversely, smooth margined leaves are found either with *MIR164* overexpression or in recessive *cuc2* mutants [18]. A role for *CUC* genes in the maintenance of phyllotaxy is seen in microRNA-resistant *CUC2* plants [19•]. Leaves are initiated correctly but, due to ectopic *CUC* expression, their growth is altered and thus the phyllotaxy is abnormal. This phenotype is also seen in the triple *MIR164a MIR164b MIR164c* mutant [17•]. Finally, reduction of *CUC2* and *CUC1* levels by overexpressing *MIR164* enhances a *cuc3* axillary meristem defect, revealing an additional role for *CUC1* and *CUC2* in this process [20].

Lateral organs — leaves

In *Arabidopsis*, *MIR165* and *MIR166* are two related microRNAs that differ at a single position but are thought to target the same set of *HD-ZIPIII* genes. In general, the overexpression of a microRNA is expected to recapitulate the loss-of-function phenotype of its targets. Now, Zhou *et al.* have found that overexpressing *MIR165* causes a phenotype different from that of *MIR166* overexpression. This phenotype matches the loss-of-function phenotype for *HD-ZIPIII* genes, an observation that may be explained if *MIR165* and *MIR166* target different genes with different efficiency [21]. If the many HD-ZIPIII proteins differ in their functions, understanding their expression patterns and the effects of their microRNA-resistant versions becomes particularly important. Itoh *et al.* [22] have carried out such a detailed study on the five members of the *HD-ZIPIII* family in rice, providing detailed expression data and setting the basis for additional functional studies.

Ori *et al.* have established the function of the *Lanceolate* (*LA*) gene of tomato in the morphogenesis of compound leaves [23••]. Normal tomato leaves are compound and consist of several orders of leaflets, while semidominant *La* mutant leaves are simple. Cloning of the gene showed that *LA* encodes a TCP transcription factor, and that the semidominant alleles carry point mutations at a *MIR319*-binding site [23••]. Interestingly, the expression domains of *LA* and *MIR319* partially overlap, suggesting that *MIR319* modulates, rather than fully downregulates, *LA* expression. Thus, the authors propose that *MIR319* and *LA* act together to regulate the competence to make leaflets, an idea supported by the simple leaves of *LA* overexpressors and the prolonged morphogenesis at the margins of *MIR319* overexpressors [23••].

MicroRNAs not only affect leaf shape but also regulate cell differentiation within the leaf. A role for a microRNA, *MIR824*, in the patterning of stomatal complexes in

Arabidopsis was recently discovered [24]. *MIR824* targets the *MADS*-box gene *AGAMOUS-LIKE16* (*AGL16*), which is normally expressed in guard cells [25]. Plants expressing a *MIR824*-resistant version of *AGL16* develop higher order stomatal complexes as a result of the early formation of meristemoids, which takes place over a prolonged period. Consistent with this function, both *MIR824* and *AGL16* are expressed in the meristemoid lineage [24]. However, they do not occur simultaneously in the same cell types; while *MIR824* was expressed in the satellite meristemoids and guard mother cells, *AGL16* was expressed in differentiated guard cells.

Characterization of leaf mutants has continued to provide mechanistic insight into the biogenesis and mode of action of microRNAs. Analysis of a classic leaf mutant, *serrate* (*se*), has revealed an unexpected convergence between processing of microRNA precursors and protein-encoding transcripts. The serrated leaf phenotype of *se* mutants is similar to that of mutants of the cap-binding complex (CBC) involved in recruitment of splicing machinery. While *SE* was previously shown to function in primary microRNA processing [26,27], use of whole genome tiling arrays uncovered an additional function for *SE* in the splicing of normal transcripts [28]. This finding raises the possibility that *SE*, along with its interacting partners *DCL1* and *HYL1* may be part of a more extensive CBC complex, or may be a mediator between the microRNA-processing complex with the CBC [28]. Leaf serration similar to that of *se* mutants was also found in *abh1* single mutants, which reinforces the link with the miRNA pathway [29]. *ABH1* encodes a subunit of a nuclear CBC. Indeed, the primary transcripts of some microRNA genes as well as miRNA targets were represented at increased levels in the *abh1* single mutant. Gregory *et al.* hypothesize that ABH1 facilitates the correct processing of microRNA transcripts by binding to their 5′-ends and directly interacting with some protein of the miRNA biogenesis complex, perhaps SE [29].

Roots and plasmodesmata

While *MIR166* plays a major role in regulating *HD-ZIPIII* transcription factors controlling vascular development and polarity in the aerial portions of the plant, it also affects lateral root initiation. Work in *Medicago* showed that *MIR166* overexpression causes a decrease in lateral root number, and consequently a decrease in the number of nitrogen fixing root nodules [30]. Work on *MIR399*, which targets the *PHO2* gene that regulates phosphate homeostasis, revealed an intriguing case of long distance microRNA transport and function from shoots to roots. Grafts of shoots overexpressing *MIR399* onto wild-type roots showed accumulation of the mature microRNA and decreased *PHO2* transcript in root tissue [31•,32•]. These results suggest that microRNAs can function noncell autonomously, in contrast to previous studies showing that microRNAs function locally only where they are expressed. Several microRNAs have been cloned from phloem sap in several species [33,34], but whether they are transported to the phloem through the cellular connections known as plasmodesmata (PD) is unknown. Recent work by Kobayashi *et al.* suggests a role for PD function in siRNA-mediated RNA silencing [35]. The *increased size exclusion limit2* (*ise2*) mutant alters PD morphology and continues to traffic large molecules at later stages of embryogenesis in contrast to normal embryos. Interestingly, *ise2* suppresses post-transcriptional gene silencing, although levels of microRNAs and siRNAs seem normal in the mutant. The *ISE2* gene encodes an RNA helicase, although it is unclear whether it regulates transcripts necessary for PD function or, like the pumpkin PSRP1 protein, it helps traffic small RNAs directly [34].

Flowering and sex determination

Work on the *MIR172* microRNA, which targets *APETALA2* (*AP2*) transcription factors, showed that it plays a major role in regulating floral meristem fate, organ identity, and flowering time [36,37]. A detailed study of *MIR172* function in flowers uncovered the mechanism behind its effects on floral meristem size and organ patterning. MicroRNA-resistant forms of *AP2* cause an enlarged indeterminate floral meristem, floral organ patterning defects, and extra whorls of stamens [36]. Zhao *et al.* showed that the floral meristem defect of these transformants is partially suppressed by *wuschel* (*wus*), but is synergistic with *agamous* (*ag*) [38••], implicating both genes in the *AP2* pathway. In addition, the inner boundary of the *B*-class genes *APETALA3* and *PISTILLATA* was enlarged and shifted in the transformants, thus explaining the extra stamen defect. These data point to a more complex model for *AP2* function, where *AP2* does not simply repress the *C*-class gene *AG* as in the classic ABC model, but also acts in separate, parallel pathways to repress *WUS* and to define the boundary of *B*-class gene expression.

In *Petunia* and *Antirrhinum* a different microRNA, *MIR169*, seems to have adopted the role of *AP2*. The *blind* mutant of *Petunia* and the *fistulata* mutant of *Antirrhinum* show homeotic transformations in which second whorl organs are staminoid [39,40]. Both mutations are deletions of *MIR169* [41•], which targets the *NF-YA* or CCAAT-box binding factor gene family [42]. These transcription factors are thought to regulate the *C*-class genes, whose expression changes in the *blind* and *fistulata* mutants [41•].

MIR172 also plays a role in the photoperiodic flowering pathway in *Arabidopsis* and sex determination in maize. Earlier studies found that *MIR172* regulates flowering time through the repression of the *AP2* genes *TOE1* and *TOE2* [37], but it was not clear how *MIR172* responds to environmental signals to regulate these genes. Recent

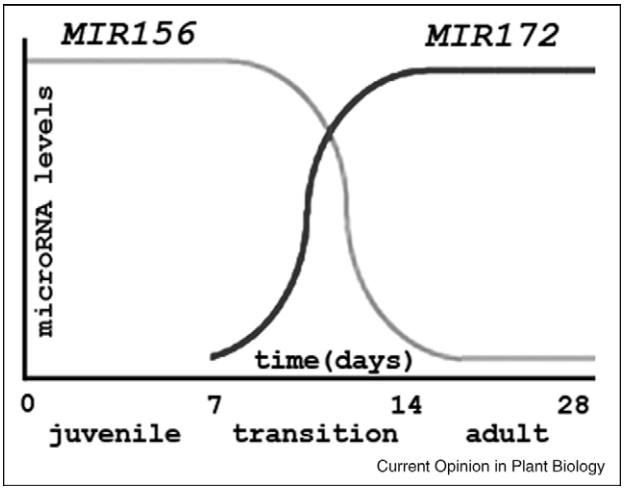
studies have found that *MIR172* levels are decreased in several photoreceptor mutants, including *phyA*, *hy4*, *fha*, and *gigantea* (*gi*) [43]. *GI* encodes a nuclear protein involved in promoting flowering and regulating circadian rhythms. *MIR172* ultimately affects flowering time through regulation of the mobile floral inducer *FT*, which acts downstream of *TOE1* and *TOE2*, but in a pathway separate from *CONSTANS* [43]. Both *DCL1* and *SE* were also downregulated in the *gi* mutant, so it is possible that the late flowering phenotype is a result of an alteration in microRNA processing.

The involvement of *MIR172* in sex determination and meristem branching in maize inflorescences was discovered by the cloning of two classic maize mutants, the recessive *tasselseed4* (*ts4*) mutant and the dominant *Tasselseed6* (*Ts6*) mutant. Both mutants belong to a class of *tasselseed* mutants that fail to abort pistils in the male inflorescence and increase indeterminate meristem branching in maize [44]. *TS4* encodes an unique microRNA of the *MIR172* family, *ZMA-MIR172E* [45•]. A target of *TS4* is the *AP2* gene *INDETERMINATE SPIKELET1* (*IDS1*), whose mutations partially suppress the *ts4* phenotype. Cloning of *Ts6* revealed it to be the same gene as *IDS1*, except with mutations within the *MIR172*-binding site. While the meristem indeterminacy defects of *ts4* and *Ts6* are similar to those caused by microRNA-resistant forms of *AP2* in *Arabidopsis*, the sex determination defect is novel. *IDS1* and a related *ts4* target gene, *SISTER OF IDS1* (*SID1*), redundantly repress the maize *C*-class MADS-box genes *ZAG1* and *ZMM2* in bracts (in press). In *ts4* and *Ts6*, these MADS-box genes may be affected by the persistent expression of *AP2* genes, and somehow allow a bypass of the sex determination signal [45•].

Timing and phase change

MicroRNAs have long been suspected of controlling the juvenile-to-adult vegetative phase transition in *Arabidopsis*, since many microRNA biogenesis mutants alter phase change [46]. In particular, the *MIR156* microRNA has been the subject of great scrutiny since the discovery that overexpression of microRNA-resistant versions of one of its target genes, *SPL3*, causes a truncation of the juvenile phase and early flowering [47]. Like *MIR172*, *MIR156* regulates these genes at the level of translation [48], which is proving to be a mode of microRNA regulation in plants more common than previously thought [49]. In addition, overexpression of *MIR156* causes late flowering and extension of the juvenile phase [47,50]. In maize, a similar phenotype is seen in the dominant mutant *Corngrass1* (*Cg1*) [51•]. Cloning of *Cg1* revealed that it encodes a tandem *MIR156* gene that is overexpressed in the mutant. Interestingly, another *MIR172* target, *GLOSSY15*, which maintains juvenile leaf cell identities, is ectopically expressed in the *Cg1* mutant [52]. This observation may be explained by the presence of reduced levels of *MIR172* in *Cg1* leaves that overexpress *MIR156* [51•]. Thus, there appears to be a converse regulatory relationship between *MIR156* and *MIR172*, where the relative balance of each may be important for the timing of the phase transition (Figure 1).

Figure 1



Reciprocal expression patterns of MIR156 and MIR172 in the juvenile and adult phase of development.

Other targets of *MIR156* play different roles in shoot development in *Arabidopsis*. For example, *MIR156* overexpression causes the overproduction of leaves and a shortened plastochron [47,50]. These phenotypes are primarily due to downregulation of *SPL9* and *SPL15* [53], which act to limit leaf initiation rates. By using tissue-specific promoters to drive *MIR156* expression, it was shown that the shortened plastochron effect could not be induced with meristem-specific expression, but only with lateral organ expression [54], indicating a noncell autonomous effect of either *MIR156* or one of its *SPL* targets on the meristem. The *SPL9* gene represents a likely noncell autonomous target since it is not expressed in the meristem, and yet can affect plastochron when *MIR156* resistant forms are expressed in either leaf primordia or the meristem [54].

There are several interesting parallels between plant and animals in the control of stage-specific developmental events by microRNAs. In *C. elegans* the transition from early larval stages to the mature adult is controlled by the sequential expression of the *lin-4* and *let-7* microRNAs [55], the functions of which appear to be conserved in other animal species [56]. Along the same lines, the transition from juvenile to adult shoot development in plants appears to be regulated by the *MIR156* and *MIR172* microRNAs, both of which appear to be sequentially expressed and conversely regulated. As in animals, both microRNAs control genes that specify cell identities specific to each stage of development. In maize, *MIR156* appears to be

present during the juvenile phase [51•] when *MIR172* is absent, and the converse is true in the adult [45•,57] (Figure 1). These reciprocal expression patterns of *MIR156* and *MIR172* appears to be conserved in *Arabidopsis* as well [37,47]. It will be critical to examine this converse relationship in other plants, and, more importantly, to associate different juvenile-to-adult transition times with the gain or loss of each microRNA. Also intriguing is the fact that both these microRNAs were found in phloem sap [33], opening the possibility that they also act noncell autonomously to influence developmental timing.

Conclusion

This review of small RNA function in plant development provides just a snapshot of the many pathways affected by these small molecules. Future work on the transcriptional regulation of these microRNAs will give a clearer picture of how they are able to regulate their targets in such a precise manner. For example, what factors are responsible for turning *MIR156* off and *MIR172* on during the transition to the adult phase? Also, the evidence that microRNAs can move through graft junctions and affect gene expression at a distance provides the implication, but not the proof, that they may act noncell autonomously. Is this a general theme for all small RNAs? As more small RNAs become sequenced from different organisms there is little doubt that many other novel processes will be found to be under the control of these multifaceted molecules.

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